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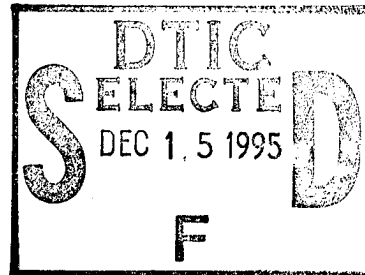
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Vaccines to Breast Cancer Based on P53 Mutants

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"A Replication-Defective Human Adenovirus Recombinant Serves as a Highly
Efficacious Vaccine Carrier"
Submitted to *Journal of Virology*

INTRODUCTION

Active cancer immunotherapy is generally based on vaccines that carry an antigen that is only (or at least preferentially,) expressed on cancer cells and not on normal, non transformed cells. Oncoproteins derived either from viruses, such as some strains of human papilloma virus, or from mutations of gene encoding proteins involved in signal transduction or transcription regulation, fulfill these requirements and might hence be employed to construct such cancer vaccines. For example, point mutations of p53 that cluster in well-defined mutational hotspots are the most commonly found molecular abnormality in human malignancies, including breast cancer. The point mutations affect the structure of the p53 protein and in consequence, its ability to bind DNA. The mutations and the associated structural changes also result in delayed degradation and overexpression of the protein. Point mutations of self proteins, such as those found in p53 oncoproteins, can result in novel epitopes that are no longer tolerated by the immune system as being part of self and subsequently activate T cell responses. In the same token, overexpression of a self protein can cause the exposure of cryptic epitopes and thus overcome tolerance. Vaccines based on mutated p53 might thus induce both responses, i.e., those against the mutated sequence that only protects against tumor cells carrying the homologous mutation as well as those against wild-type sequences that might cross-react with tumor cells expressing a variety of mutations. The later response might be induced more readily as Ir-gene control will most likely govern the response against most of the single point mutations.

To reiterate, the long-term goal of the application is to develop vaccines based on mutated p53 that can induce protective immune responses to already established tumors. In this application, prototype vaccines based on expression vectors, recombinant viruses, or purified proteins will be developed and tested in a mouse model for their efficacy in limiting the spread of tumors carrying homologous or heterologous p53 mutations.

BODY

In the first year of this application we generated a number of different vaccines and started their in vitro characterization. In addition, we started to develop a mouse tumor model to test the efficacy of the vaccines.

1. Generation of prototype vaccines

a) Vaccinia virus recombinants. We initially cloned a truncated form of two p53 genes, one carrying a mutation in position 135 (Ala to Val), the other in position 234 (Met to Ile), into the pSG520T transfer vector. The truncation was designed to express amino acids 1-343 of the protein which include the mutational hotspots but lack the oligomerization domain that is required for the transforming phenotype of mutant p53. Upon recombination with vaccinia virus and plaque purification of a number of recombinants (of which in general, using the method described in more detail below, at least 50% carry the introduced insert,) we realized that none of the available antibodies to wild-type or mutant mouse p53 recognized this truncated protein. We thus recloned the full-length p53 genes (one expressing a single mutation of Ala to Val in position 135; the other expressing two mutations, one at position 168, i.e., Glu to Gly, and one in position 234, i.e., Met to Ile) into the pSC11 multicloning site. The pSC11 vector carries, (as opposed to the pSG520T vector,) a lacZ gene, thus greatly facilitating the identification of recombinant viruses. CV-1 cells were transfected with the pSC11.p53 transfer vectors and then infected with 10^{-1} pfu of vaccinia virus strain Copenhagen per cells. Forty-eight

hours later cells were freeze-thawed 3 times, and the cell free supernatants were used to infect tk⁻ cells in presence of trifluorothymidine or BudR. Cells were overlaid four hours later with LMB agarose. After forty-eight hours, when viral plaques became visible, plates were overlaid with an additional layer of agarose containing X-gal. Blue plaques became visible after an overnight incubation at 37°C. Several plaques were harvested for each construct. The virus was expanded and then used to infect p53 negative embryonic cells. Twenty-four hours later cells were fixed with acetone and then incubated with pAb421 (a monoclonal antibody to p53)(1). Cells were washed, and after an additional incubation with an FITC-labeled goat anti mouse Ig, were visually assessed for staining. Viruses that resulted in positive staining were expanded for further studies.

b) Recombinant adenovirus. In our original application we proposed to use vaccinia virus recombinants for our studies. In the meantime, results that we obtained in a viral system (see Appendix) showed that recombinants based on an E1 and E3 deleted. Human strain 5 adenovirus is more potent in inducing cytolytic T cell responses when compared to vaccinia virus recombinants. We thus decided to include adenoviral recombinants in our studies. We cloned the two p53 genes carrying the 135 mutation or the 234/168 double mutation into the pAdCMV transfer vector (2) which is designed to express the insert under the control of the CMV regulatory element within the E1a gene of adenovirus, thus rendering the virus replication defective. In order to generate recombinant virus, 292 packaging cells (3) that carry the E1 gene of adenovirus were co-transfected with the transfer vector and purified, ClaI-cut adenoviral DNA. Cells were subsequently overlaid with agarose (4). Plaques were picked 6-8 days later and expanded. We are currently in the process of testing the recombinants for expression of p53 upon infection of p53 negative cells.

c) DNA vaccines (5). We tested 2 of the pSV2gpt based vectors expressing p53 mutants for induction of cytolytic T cells in mice. Neither gave a positive response. Mounting evidence suggests that strong promoters are required for the induction of T/B cell responses by genetic immunization. The SV40 promoter that is present in pSV2gpt is a weak viral promoter when compared to the CMV promoter that, dependent on the host cell types, causes up to 100 fold higher expression. We are thus in the process of recloning the p53 genes into the pCDNA3 vector that expresses proteins under CMV control.

d) Baculovirus recombinants. We cloned the genes encoding two different mutant p53 molecules (135 mutation and 234 mutation) into the pVT-Bac transfer vector. The transfer vector was co-transfected with purified *Autographa californica nuclear polyhedrosis* virus DNA into *Spodoptera frugiperda*. Recombinant baculovirus was selected and plaque purified.

2. Tumor model

We transfected the p53 loss-mutant cell line termed (10)1 (6) with expression vectors for mutant p53 and pSV2neo. Colonies that showed positive expression for p53 were selected and supertransfected with a retroviral vector (7) expressing *v-ras* and *v-myc*. Our previous studies had shown that this combination of oncogenes leads to focus formation of cells. Within a week after the second transfection, cells were inoculated into nude BALB/c mice. Solid tumors became apparent close to the site of inoculation ~ 2 months later. The mice were euthanized, the tumors were excised, cut into small pieces and trypsinized for two hours at room temperature to isolate single cells. Cells were expanded and expression of p53 was reassessed and found to be positive. Cells were then inoculated into immunocompromised mice as well immunocompetent BALB/c mice. Immunocompromised mice developed tumors while the BALB/c mice failed to show any tumor growth indicating that the tumor cells had induced a protective immune response.

With the help of Dr. B. Knowles, (Bar Harbor, Maine,) we transformed embryonic cells from p53 knock-out mice (8) with SV40. Several lines from different organs (lung, kidney, heart, thymus) were obtained. We are currently transfecting some of the lines with expression vectors for mutant p53 using a plasmid conferring hygromycin resistance for co-transfection. Transfected cells will be tested for expression of p53. Clones that express p53 will be injected into SV40 large T transgenic mice (male mice that are sterile will be provided to us by Dr. B. Knowles) which should be tolerant to SV40 and thus allow growth of the tumor cells (9,10).

CONCLUSIONS

We decided to change some of the experimental approaches that were described in the original application.

We proposed to use truncated p53 for most of our vaccine preparations. As none of the antibodies available recognized the truncated protein we are now preparing vaccines that express the full-length protein. These vaccines will be used to establish T cell assays which in turn will be used to identify vaccines expressing truncated p53.

We added an additional recombinant viral vaccine based on an E1 and E3 deleted adenovirus strain 5. We decided to include this type of a recombinant vaccine in our studies for the following reasons:

- 1) results from the rabies virus system showed that this recombinant is an excellent inducer of cytolytic T cell responses that we assume play a major role in limiting the spread of most cancers.

- 2) Due to its inability to replicate, the adenovirus is much safer for use in humans compared to vaccinia virus recombinants and

- 3) the adenovirus will facilitate some of our immunological studies. For restimulation of cytolytic T cell responses, the highly cytopathic vaccinia virus is problematic, while adenoviral recombinants that do not cause cell death were shown to cause good in vitro reactivation of T cells.

We changed the tumor model. We initially proposed to use transfected (10)1 cells in BALB/c mice. Although we were able to generate mutant p53 expressing (10)1 cell lines that formed tumors in immunocompromised mice, these tumors could not be transplanted to immunocompetent mice. We will follow two steps to rectify this problem:

- 1) we transformed embryonic cells from p53 knock-out mice with large T of SV40. We are stably transfecting these cells with expression vectors for p53 mutants in the presence of a hygromycin selection marker. p53 expressing clones will be injected into immunocompromised mice. Tumors and cell lines thereof will be isolated and transplanted into SV large T transgenic mice. Provided that these cells form tumors in the SV40 large T transgenic mice we will continue using this as our tumor model.

- 2) Dr. T.J. McDonnell (U. of Texas) has isolated a number of tumors from mice transgenic for bcl-2 and myc (11). All of these tumors have p53 mutations that were sequenced. Dr. T.J. McDonnell is willing to provide us with these lines (that are derived from mice of the H-2^b background back-crossed onto C57Bl/6). We will use these cell lines as an alternate (or additional) model.

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